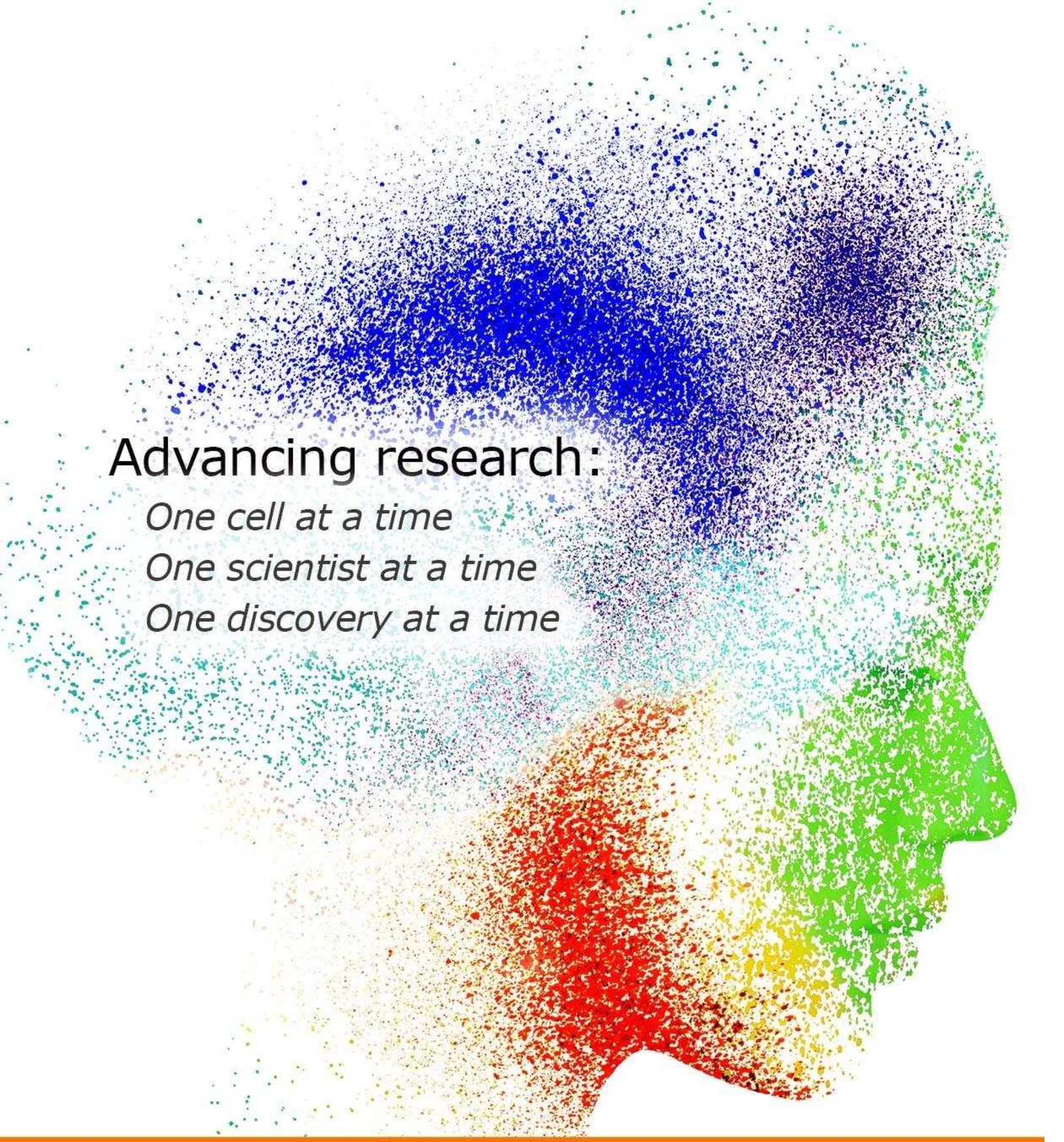

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ECTOPIC EXPRESSION OF RET RESULTS IN MICROPHTHALMIA AND TUMORS IN THE RETINAL PIGMENT EPITHELIUM

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The retinal pigment epithelium (RPE) is essential for eye development by interacting with the overlaying neuroepithelium. Regulatory sequences of the gene encoding for tyrosinase-related protein 1 (TRP-1), linked to the *lacZ* reporter gene, lead to strong and specific β-galactosidase expression in the RPE. We asked how the oncogene *ret* would affect this epithelial cell type during mouse development. We used the TRP-1 promoter to express ret in the developing RPE, and obtained transgenic mouse lines, which showed mild to severe microphthalmia. During development, the RPE changed to a stratified epithelium with reduced or absent pigmentation from E10.5 onward. In addition, proliferation of RPE cells and tumor formation were observed from E12.5 onward. These early events prevent closure of choroid fissure and lead to microphthalmia and secondary malformations after birth. We conclude that *ret* transgene expression in the RPE prevents normal differentiation of this epithelial layer and induces proliferation and tumor formation. The appearance of the microphthalmic phenotype underlines the requirement of a normally developed RPE for eye development. *Int. J Cancer* 80:600–605, 1999.
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The optic cup is a derivative of the prosencephalic neuroepithelium and originally comprises 2 layers. The inner layer gives rise to the multilayered neuroretina and the outer layer forms the retinal pigment epithelium (RPE), which consists of a single layer of pigment cells (for review see Zhao *et al.,* 1997). Within the RPE, tyrosinase and tyrosinase-related proteins 1 and 2 (TRP-1, TRP-2) are important for pigmentation and are involved in the biosynthesis of melanin. These 3 genes are also expressed later in development in pigment cells derived from the neural crest, the melanocytes. The promoters of tyrosinase, TRP-1 and TRP-2 all direct transgene expression to the developing RPE, starting at about E9.5 (TRP-2) or E10.5 (tyrosinase and TRP-1) (MacKenzie *et al.,* 1997; Raymond and Jackson, 1995; Tief *et al.,* 1996). The TRP-2 promoter appears to be the strongest and earliest active, with expression in melanoblasts detected from the time of their appearance at E10.5 (MacKenzie *et al.,* 1997). In contrast, expression from the TRP-1 promoter is nearly undetectable in melanocytes of transgenic mice (Penna *et al.,* 1998; Raymond and Jackson, 1995), in contrast to its efficient expression in developing RPE. Transcription factors such as Otx-2 or microphthalmia (Mi) are specifically expressed in the RPE and have been shown to be important for differentiation and expression of pigmentation genes. In the adult, the RPE is essential for function of photoreceptors in the neuroretina. The RPE provides physical protection, regulates the flow of substances to and from the neuroretina and phagocytozes shed photoreceptors. Image sharpness is improved by the presence of the pigment melanin, leading to absorption of light penetrating the sclera. Ablation of the RPE, as demonstrated in transgenic mice, interferes with and blocks differentiation and maintenance of the neuroretina (Raymond and Jackson, 1995).

Different mouse mutations cause degeneration of the neuroretina, as has been described for *rd* (retinal degeneration) or retinitis pigmentosa (Humphries *et al.,* 1997). Defects in the RPE have been reported in a mouse model for gyrate atrophy. Here, deficiency of ornithine δ-aminotransferase (OAT) led to progressive retinal degeneration over the first year of life with accumulation of phagosomes and crystalloid inclusions in the RPE (Wang *et al.,* 1996). In mice carrying mutations of the Mi gene, retinal degeneration, reduced eye pigmentation and small eyes are also observed (Moore, 1995). The Mi protein is a transcription factor of the basic helix-loop-helix-leucine zipper family, which binds DNA as homo- or heterodimer with members of the same transcription factor family (Moore, 1995). It is essential for differentiation of pigmented cells including the RPE, and is able to activate transcription of *tyrosinase* and *TRP-1* genes (for review see Zhao *et al.,* 1997).

The *RET* proto-oncogene encodes a receptor tyrosine kinase, which was originally identified as a transforming gene by transfection of T-cell lymphoma DNA into NIH3T3 cells (Takahashi *et al.,* 1985). RET is expressed in a variety of cell lineages in the developing central and peripheral nervous system and the excretory system. Mice homozygous for targeted deletion of the *RET* locus die soon after birth. This revealed that RET is essential for renal organogenesis and enteric neurogenesis (Schuchardt *et al.,* 1994). In humans, mutations of RET are responsible for the MEN2 syndromes (multiple endocrine neoplasia type 2A and type 2B, familial medullar thyroid carcinoma, medullar thyroid carcinoma) and Hirschsprung disease. These diseases are regarded as neurocristopathies, since they affect neural crest-derived cells and their derivatives. However, there is also evidence for effects on other cell lineages, as demonstrated by parathyroid hyperplasia in MEN2A patients (for review see Edery *et al.,* 1997). A receptor complex comprised of TrnR1 [transforming growth factor β-related neurotrophic factor 1, glial cell line-derived neurotrophic factor receptor α (GDNFRα)] and RET has recently been identified and found to be capable of mediating signaling of both GDNF and neurturin. An additional coreceptor, TrnR2, can replace TrnR1 in this receptor complex (*e.g.,* Baloh *et al.,* 1997). *In vitro* expression of an epidermal growth factor (EGF) receptor/RET chimeric protein has revealed that the cytoplasmic domain containing the tyrosine kinase activity is sufficient to provide RET-specific signaling (Santoro *et al.,* 1994).

Several RET fusion proteins have been isolated, all of which are dominant oncogenic forms of RET, *i.e.,* RET/PTC1, RET/PTC2, RET/PTC3. These fusion proteins originate from somatic rearrangements of the cytoplasmic tyrosine kinase domain of RET with

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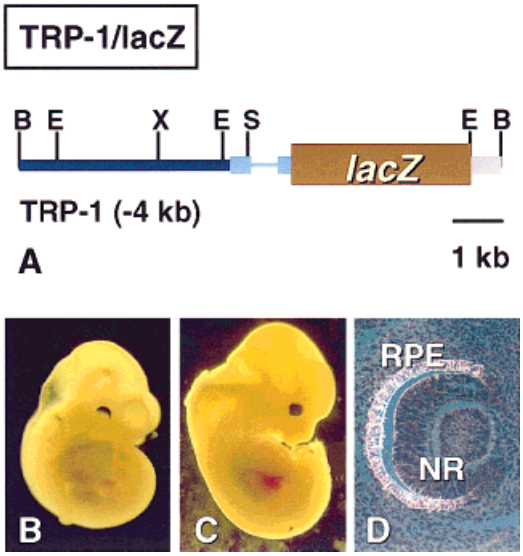

FIGURE 1 – A *TRP-1/lacZ* fusion gene in transgenic mice. *(a)* The *TRP-1/lacZ* fusion construct. B = BamHI, E = EcoRI, X = XbaI, S = SalI. *(b,c)* E10.5 embryo *(b)* and E12.5 embryo *(c)* transgenic for TRP-1/lacZ and stained with Xgal. *(d)* Section through eye of an E12.5 embryo, transgenic for TRP-1/lacZ and stained with Bluogal. Expression from TRP-1 promoter is restricted to the developing RPE. No signal is seen in the neuroretina (NR).

different N-termini (H4, RIα and ELE1). Following dimerization of the N-termini, RET tyrosine kinase becomes constitutively activated (for review see Edery *et al.,* 1997). The *ret* transforming gene is a fusion gene of the human *RET* proto-oncogene and the zinc finger-containing *rfp* gene (RET finger protein), a transcriptional activator, and arose as rearrangement in a transfection assay (Cao *et al.,* 1997; Takahashi *et al.,* 1985). The ret chimeric protein has also been proposed to function as constitutively activated RET tyrosine kinase, caused by ligand-independent dimerization (Cao *et al.,* 1997). We used this oncogene to directly affect the RPE, and asked if and how it would act on this specialized cell type. To target ret expression to the RPE, we used 4 kb of the promoter of the gene encoding TRP-1, a protein involved in pigment synthesis (Raymond and Jackson, 1995). We first showed by using a reporter gene that this promoter specifically targets expression to the RPE in transgenic mice. Expression of *ret* transforming gene in the RPE results in microphthalmia and tumor formation. The changes in the RPE, which is thickened and largely unpigmented, severely affect the development of the eye.

MATERIAL AND METHODS

TRP-1/lacZ fusion construct

The TRP-1 first intron was isolated by polymerase chain reaction (PCR) (primer 1: 5′-agagtcgacctggcatcaggggaaaagc-3′; primer 2: 5′-agacctcgagtcttctcgagcaagagtt-3′), which placed a SalI site 5′ and a XhoI site 3′ to the intron. This fragment was inserted into the SalI site within the pUC polylinker of the lacZ reporter vector pSVOD19a (a gift from Dr. E. Blair). The TRP-1 leader sequence

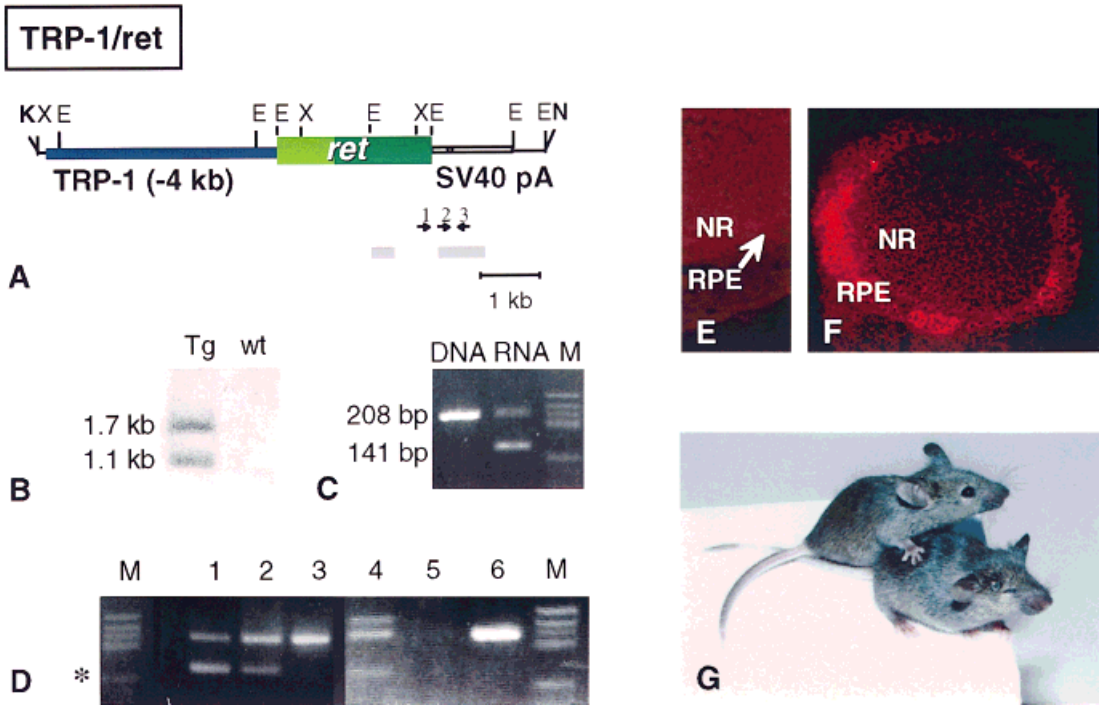

FIGURE 2 – Transgenic mice carrying a *TRP-1/ret* fusion gene. *(a)* Scheme of the *TRP-1/ret* fusion gene used for microinjection. The construct contains 4 kb TRP-1 promoter, the *ret* transforming gene and SV40 small T splice and polyadenylation sequences. Probes used in Southern blot are indicated by gray bars. Location of PCR primers 1, 2 and 3 is depicted by arrows. K = KpnI, X = XhoI, E = EcoRI, N = NotI. *(b)* Southern blot analysis of a TRP-1/ret transgenic mouse (Tg). EcoRI digests of tail DNA were hybridized [probes in *(a)*] resulting in a 1.1 kb fragment and a 1.7 kb fragment. wt = wild type (non-transgenic littermate). *(c,d)* Analysis of transgenic expression. PCR analysis of DNA and RNA of TRP-1/ret transgenic mouse using primers 2 and 3, on both sides of the SV40 small T intron. Amplification of genomic DNA results in a longer fragment (208 bp). RT-PCR from total RNA of TRP-1/ret transgenic eye results in a shorter fragment [141 bp; depicted by asterisk in *(d)*]. Transgenic expression is seen in adult eyes (lanes 1,2) and E12.5 embryo (lane 4). M = molecular weight marker V (Boehringer Mannheim, Germany). Lanes 3,6: Genomic DNA, transgenic. Lane 5: RNA, E12.5 embryo, non-transgenic. *(e,f)* Eye sections (E12.5 embryo) analyzed by immunofluorescence for expression of ret. No distinct signal is recognized in eye of wild-type littermate *(e),* but detected in RPE of transgenic embryo *(f).* NR = neuroretina. *(g)* Microphthalmia in TRP-1/ret transgenic mice: Wild-type mouse (left) and transgenic littermate (right, 3 weeks old, line 152).

and promoter extends between a BamHI site located around 4 kb upstream of the transcription initiation site and a PvuII site at +107. The entire TRP-1 promoter and leader sequence was placed in pSVoD19a upstream from the TRP-1 first intron. A residual BamHI site located in the pSVoD polylinker was destroyed by partial digestion with BamHI followed by treatment with Klenow. The entire TRP-1/lacZ construct was excised as a BamHI fragment.

TRP-1/ret fusion construct

A HindIII-PvuI fragment of construct MT/ret (Iwamoto *et al.,* 1991) (kindly provided by Dr. T. Iwamoto) containing 2.7 kb of ret cDNA and 1.7 kb of SV40 small T splice and polyadenylation sequence was cloned into HindIII and EcoRV sites of pBluescript IISK− (Stratagene, La Jolla, CA). A 4 kb BamHI fragment of the murine TRP-1 promoter, extending up to +107 of the *TRP-1* gene (kindly provided by Dr. I. Jackson), was inserted 5′ to ret into the HindIII site. The construct was deliberated from vector sequences by digestion with KpnI and NotI.

Generation and analysis of transgenic mice

Transgenic constructs were released from vector sequences by cleavage with BamHI (TRP-1/lacZ) or KpnI and NotI (TRP-1/ret), purified from agarose gels using silica powder and injected into fertilized oocytes derived from mating of CB6 (BALB/c × C57BL/6J) F_1 females and males, or NMRI mice (obtained from Harlan, Horst, The Netherlands). Production of transgenic mice followed established procedures. They were identified by Southern blot analysis or PCR analysis of genomic DNA isolated from tail biopsies (Schmidt *et al.,* 1998). For lacZ, we used a probe derived from lacZ coding sequence (not shown), and for PCR analysis, a sense primer located at the 5′ end of exon 2 of TRP-1 (5′-agagcagcataggagacc-3′, position 1186–1205) and an antisense primer in the *lacZ* gene; 2 of 4 identified founders (822 and 825) transmitted the transgene, and transgenic lines were established by breeding to non-transgenic NMRI mice. β-Galactosidase staining of embryos was performed essentially as described using Xgal or Bluogal (Schmidt *et al.,* 1998).

For genotyping of TRP-1/ret transgenic mice by Southern blot analysis, we used a probe derived from SV40 splice and polyadenylation sequence or an EcoRI-BglII fragment of *ret* cDNA (Iwamoto *et al.,* 1991). PCR analysis was performed using an upstream primer in *ret* (P_1, 5′-tcccttccacatggattgaa-3′; position 2354–2373 of *ret*) and a downstream primer (P_3) 3′ to the SV40 intron (5′-ggcatttcttctgagcaa-3′, position 4489–4506 of SV40 DNA). Using PCR and/or Southern blot analysis, a total of 9 founders were identified (Table I). For RT-PCR analysis, total RNA was prepared from adult eyes or embryonic head and reverse transcription (RT)-PCR was performed as described (Schmidt *et al.,* 1998) using primer P_3 and an upstream primer (P_2) 5′ to the SV40 intron (5′-cttacttctgtggtgtga-3′, position 4696–4679 of SV40 DNA).

To generate TRP-1/ret transgenic mice on a $Kit^{W\text{-}v}/Kit^{W\text{-}v}$ mutant background, $Kit^{W\text{-}v}/+$ females (obtained from Jackson Laboratory, Bar Harbor, ME) were mated to TRP-1/ret transgenic males. $Kit^{W\text{-}v}/+$ TRP-1/ret transgenic mice were further bred to $Kit^{W\text{-}v}/+$ mice. The different $Kit^{W\text{-}v}$ genotypes were distinguished by phenotype ($Kit^{W\text{-}v}/+$: white spotting on belly; $Kit^{W\text{-}v}/Kit^{W\text{-}v}$: black-eyed white).

TABLE I – TRP-1/RET TRANSGENIC MOUSE LINES DEVELOP MICROPHTHALMIA AND RPE TUMORS[1]

Line number	Copy number	Eye phenotype	RPE tumor formation
3	5–10	Closed eyelids, sM	+
4	ND	Enlarged eyes in founder, offspring sM with closed eyelids	ND
34	2	Enlarged eyes, some offspring with mM	+
151	3–5	Lids open, mM	+
152	10–20	Closed eyelids, sM	+
161	5–10	Closed eyelids, sM	+

[1]Three other lines (38, 42, 153) showed no obvious phenotype. sM = severe microphthalmia, eyes always closed; mM = mild microphthalmia, lids open; ND = not determined.

Histology

Embryos were obtained from timed breedings to $CB6F_1$ mice, with the morning of the appearance of a vaginal plug considered E0.5. They were removed from the surrounding decidua, fixed in 4% paraformaldehyde, 1× phosphate-buffered saline (PBS) and embedded in methacrylate (immunobed kit solution, Polyscience, Warrington, PA); 6–7 μm sections were cut and stained with Giemsa (Merck, Darmstadt, Germany). Sections from eyes of newborn or 7-day-old mice were treated accordingly. Sectioning of Xgal- or Bluogal-stained embryos was performed as described (Schmidt *et al.,* 1998; Tief *et al.,* 1996). For immunofluorescence, cryostat sections were fixed for 3 min in ice-cold acetone, blocked in PBS/1% bovine serum albumin (BSA) and incubated with primary antibodies. TRP-1 and tyrosinase were detected using antibodies αPEP1 and αPEP7 (kindly provided by Dr. V. Hearing). For ret we used anti-ret(tk) antibody generated against the tyrosine kinase domain of human ret (kindly provided by M. Santoro). For detection of Mi protein, we used a polyclonal antibody kindly provided by H. Arnheiter. Goat anti-rabbit CY3 antibody was used as secondary antibody (Jackson ImmunoResearch, West Grove, PA).

RESULTS

TRP-1 promoter directs expression to the developing RPE in transgenic mice

To investigate the TRP-1 promoter in transgenic mice and to compare its activity with the tyrosinase promoter, a *TRP-1/lacZ* fusion gene was constructed containing 4 kb of TRP-1 upstream sequences (Fig. 1). Two stable transgenic lines were established and analyzed. Consistent and reproducible expression was observed (Fig. 1) in the developing eye, from E10.5 onward (Raymond and Jackson, 1995). Expression of β-galactosidase was localized in the outer layer of the optic cup, the presumptive RPE (Fig. 1). Compared with tyrosinase-lacZ transgenic mice, the signal was much more intense. We therefore suggest that 4 kb of the TRP-1 promoter provides specific and strong transgene expression in the developing RPE.

Microphthalmia in TRP-1/ret transgenic mice

Ret sequence (derived from MT-ret, Iwamoto *et al.,* 1991) was ligated to 4 kb TRP-1 promoter sequences and used for generation of transgenic mice (Fig. 2*a*). Nine transgenic mouse lines were identified by PCR or Southern blot analysis (Fig. 2*b,c,* Table I). Transgenic ret expression (hereafter: TRP-1/ret) was analyzed by RT-PCR using intron-spanning primers, and detected in embryos and eyes of adult mice (Fig. 2*c,d*). To verify that TRP-1/ret transcripts result in protein expression in the developing RPE, we performed immunofluorescence staining on eye sections. Specific staining was detected in the RPE of a transgenic embryo (Fig. 2*f*) and absent in the RPE of a non-transgenic littermate (Fig. 2*e*). Four founders (3, 151, 152, 161) exhibited microphthalmia and closed or slightly closed eyelids (Fig. 2*g*). They were bred to generate stable lines. Founder 4 exhibited increased eye size, but all transgenic offspring of founder 4 were microphthalmic. Offspring of line 34 were macrophthalmic with eye size increasing in adulthood. However, mating of transgenic males and females resulted in mice with a microphthalmic phenotype. It is feasible that low expression or delayed expression of TRP-1/ret is not sufficient to interfere with early eye development, but is nevertheless capable of influencing retina and eye development at later stages. We have observed the microphthalmic phenotype reproducibly in several independent lines, and further concentrated on these lines. We conclude that expression of TRP-1/ret in the RPE of transgenic mice results in microphthalmia.

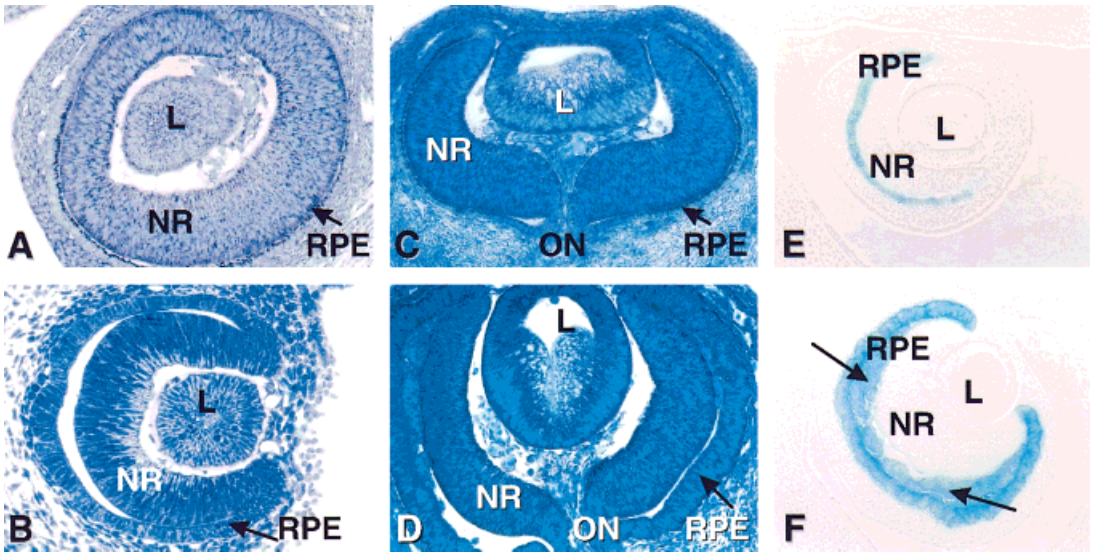

FIGURE 3 – Development of RPE in TRP-1/ret transgenic mice. *(a)* E10.5, wild type. *(b)* E10.5, TRP-1/ret transgenic. *(c)* E12.5, wild type. *(d)* E12.5, transgenic. *(e)* E12.5, TRP-1/lacZ transgenic embryo, stained with Xgal. *(f)* E12.5, double transgenic (TRP-1/ret and TRP-1/lacZ) littermate, stained with Xgal. Arrows indicate proliferations into intraretinal space positive for β-galactosidase activity. NR = neuroretina, L = lens, ON = optic nerve.

Ret affects development of the RPE and induces proliferation

Further analyses on eye development and RPE were performed on lines 3, 152 and 161, which exhibit similar microphthalmic phenotype (Table I). In these transgenic mouse lines, no alterations in skin or internal organs were observed. In wild-type mice, the developing RPE is composed of one cell layer (Fig. 3*a,c*). In the transgenic eye, already at E10.5 the presumptive RPE is thickened and consists of more than one layer of elongated cells (Fig. 3*b*). At E12.5, RPE cells proliferated into the intraretinal space, most often in the region of the presumptive ciliary epithelium. At this stage, the RPE had changed to a thick and stratified epithelium, with 2 or more cell layers (Fig. 3*d*). The presence of the tumor and/or the changes in the RPE might prevent closure of the choroid fissure and thereby cause failure to build up intraocular pressure. This might explain the development of the microphthalmic phenotype. The proliferation is originating from the RPE, as apparent from embryos bitransgenic for TRP-1/lacZ and TRP-1/ret, which were stained for β-galactosidase activity (Fig. 3*e,f*). In the RPE of E12.5 embryos, TRP-1/lacZ expression is detected in the stratified epithelium of the transgenic RPE and in the proliferating cells that are in continuation with the RPE. This furthermore demonstrates that the TRP-1 (−4 kb) promoter, which regulates expression of both lacZ and ret, is still active at this developmental stage, even though pigmentation and expression of pigmentation genes might already be affected (see below). In E15.5 embryos, the tumor is much more prominent and pigmented (not shown). The RPE is still present and recognizable as stratified epithelium with absent or reduced pigmentation.

We asked whether expression of melanogenic enzymes is affected in early eye development. In the thickened and stratified epithelium, pigmentation is reduced or absent (Figs. 3, 4). Pigmentation was only visible in some RPE cells. Tyrosinase and TRP-1, which are enzymes involved in melanin biosynthesis, are present in the RPE of non-transgenic littermates (Fig. 4*b,c*). In the transgenic RPE, both proteins were only detected in still pigmented cells, but absent in unpigmented RPE (Fig. 4*e–g*). The transcription factor microphthalmia (Mi), which is important for RPE development and is involved in regulation of tyrosinase and *TRP-1* gene transcription (for review see Zhao *et al.,* 1997), was detected in both transgenic and non-transgenic RPE (Fig. 4*d,h*). We therefore

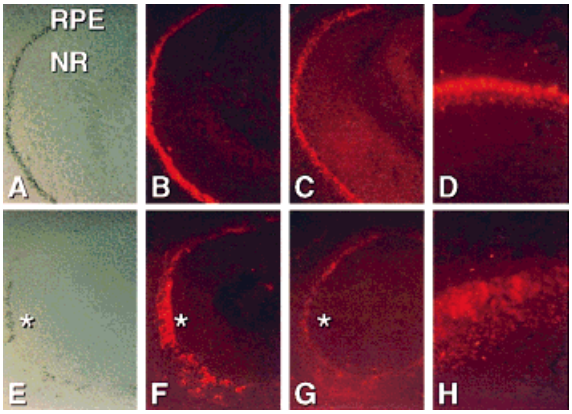

FIGURE 4 – Expression of tyrosinase and TRP-1 is affected by ret expression. *(a–d)* E12.5, wild type. *(e–h)* E12.5, TRP-1/ret transgenic littermate. Eye sections [brightfield *(a,e)*] were analyzed by immunofluorescence for expression of tyrosinase *(b,f),* TRP-1 *(c,g)* and Mi protein *(d,h).* Tyrosinase and TRP-1 expression is reduced or absent in the transgenic RPE, and maintained in cells that are pigmented [asterisks in *(e–g)*]. NR = neuroretina.

suggest that the effects on pigmentation and expression of TRP-1 and tyrosinase are not due to decreased expression of Mi.

Tumor formation after birth

Proliferation of RPE was also visible after birth in newborns (Fig. 5*a,b*) and in 7-day-old mice (Fig. 5*c–f*). In these mice, the resulting tumors were in continuation with the RPE and were often heavily pigmented (Fig. 5*f*). In older mice, tumors increased in size (not shown), but remained benign, and no metastases have been detected to date. In 7-day-old mice, the neuroretina was folded and, at some places, formed double layers with 2 ganglion cell layers directly facing each other (Fig. 5*d*). All layers had formed in the neuroretina (Fig. 5*e*). This is striking since the RPE itself was already affected early in development and was still present as unpigmented thickened epithelium (Fig. 5*d,e*). No alterations in the choroidal layer were detected in newborns or 7-day-old mice. In

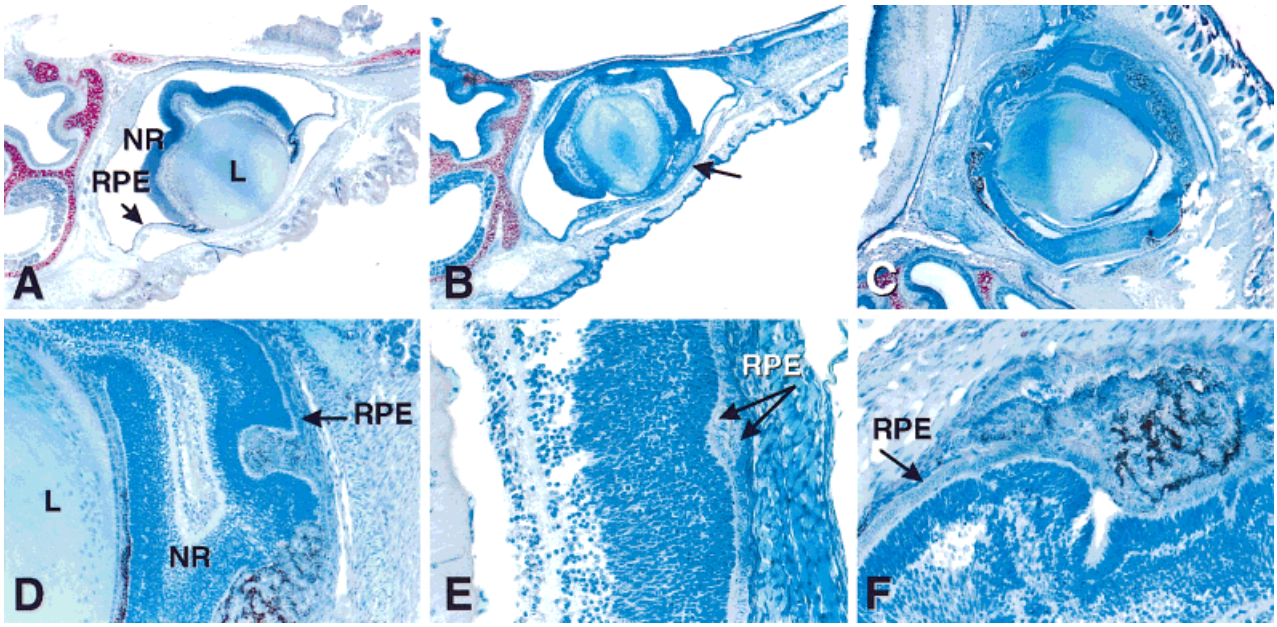

FIGURE 5 – RPE and eye are affected after birth. *(a)* Newborn, non-transgenic. *(b)* Newborn, TRP-1/ret transgenic littermate. Arrow depicts tumor formation in front of eye. *(c–f)* Seven days, TRP-1/ret transgenic eye with tumor formation and folding of neuroretina. *(d)* Magnification of *(c)* showing folding of neuroretina. *(e)* Section through neuroretina showing 2 layers of unpigmented RPE and apparently normal organization of the neuroretina. *(f)* Magnification of *(c)* showing pigmented RPE tumor. NR = neuroretina, L = lens.

addition, eye tumors were also observed in TRP-1/ret transgenic mice (line 161), which had been bred onto a $Kit^{W\text{-}v}/Kit^{W\text{-}v}$ mutant background (not shown). The $Kit^{W\text{-}v}$ mutation is characterized by deficiency in neural crest-derived melanocytes (MacKenzie *et al.,* 1997). This gives further evidence that the tumor is derived from the RPE. We thus conclude that ret expression affected the structure of RPE and induced proliferation of RPE cells in the developing mouse eye. Consequently, this leads to RPE tumor formation and severe malformation of the eye after birth.

DISCUSSION

Tyrosinase, TRP-1 and TRP-2 are expressed in melanocytes derived from the neural crest and in the RPE. The tyrosinase promoter has been used successfully in transgenic mouse experiments and leads to transgenic expression in pigment cells. The expression levels are rather low and experiments using tyrosinase-lacZ fusion constructs, which contained 6 kb of 5′ regulatory sequence, have shown that this promoter is also active in the developing and adult forebrain (Schmidt *et al.,* 1998; Tief *et al.,* 1996). The TRP-2 (Dct, dopachrome tautomerase) promoter was analyzed in TRP-2/lacZ transgenic mice and has been demonstrated to efficiently target expression to both RPE and melanoblasts/melanocytes. It is also expressed in the developing telencephalon (MacKenzie *et al.,* 1997). Analysis of a *TRP-1/lacZ* fusion gene in transgenic mice clearly showed that 4 kb of the TRP-1 promoter is sufficient for specific and high expression of the transgene in the developing RPE (Fig. 1; Raymond and Jackson, 1995). Even though TRP-1 is expressed in melanocytes, we did not detect any β-galactosidase expression in melanocytes, which suggests the existence of further regulatory elements not retained within the 4 kb promoter. Nevertheless, this specific expression pattern renders the promoter extremely suitable to target transgenes to the RPE.

In the TRP-1/ret transgenic mice, expression of the transgene was observed in the developing RPE and the eye tumor (Fig. 2). Additional evidence suggests that the effects are caused by expression in RPE only and that transgenic expression is absent or very low in melanocytes: (1) no β-galactosidase expression was detected in melanocytes of TRP-1/lacZ transgenic mice (Fig. 1; Raymond and Jackson, 1995); (2) microphthalmia and tumors were observed in several independent TRP-1/ret transgenic mouse lines (Table I), thus excluding a possible effect by the transgenic insertion site; (3) the phenotype is seen in embryonic eyes (*e.g.,* Fig. 3), at an embryonic age when choroidal melanocytes are not yet present; (4) TRP-1/ret expression was not able to compensate for kit deficiency ($Kit^{W\text{-}v}/Kit^{W\text{-}v}$) in melanocytes in 2 different transgenic lines (161, 34) which showed tumors and eye phenotype (not shown). Such a rescue of $Kit^{W\text{-}v}$-mutant melanocytes has been reported in MT/ret transgenic mice (Iwamoto *et al.,* 1991).

When targeting the *ret* oncogene specifically to the developing RPE, the observed phenotypes in the eye (besides tumor formation) are reminiscent of mutations at the Mi gene locus. In mice, at least 17 mutant alleles of microphthalmia are known. All of them show defects in neural crest-derived melanocytes, and some also in the RPE (Moore, 1995; Zhao *et al.,* 1997). From the resemblance of the phenotypes between the TRP-1/ret transgenic mice and the described mutations at the microphthalmia locus (microphthalmic phenotype, thickened RPE, decreased pigmentation) we speculated that ret interferes with activity of the Mi transcription factor in the RPE. However, Mi immunoreactivity was still detected while pigment production was reduced. Thus, the decreased pigmentation and the morphological change of the RPE seem not to be caused by reduced expression of Mi.

In humans, dominant gain-of-function mutations of RET are associated with multiple endocrine neoplasia and familial medullary thyroid carcinoma (Edery *et al.,* 1997). When targeted to transgenic mice, RET/PTC1 resulted in tumor formation (Portella *et al.,* 1996; Santoro *et al.,* 1996). Tumorigenic activity of *ret* has been reported in MT/ret, MMTV/ret and Eμ/ret transgenic mouse lines, and led to adenocarcinomas of mammary and salivary glands (Iwamoto *et al.,* 1990), melanocytic tumors (Iwamoto *et al.,* 1991) or pro-B-cell leukemia (Wasserman *et al.,* 1998). In TRP-1/ret, the tripartite motif of the *rfp* (ret finger protein) gene, which is involved

in protein-protein interaction and intracellular localization, is still retained (Cao *et al.,* 1997). It is assumed that ret (rfp-ret) causes cellular transformation by activating ret kinase activity, thus leading to proliferation. Alternatively, nuclear localization of the *rfp-ret* fusion gene might lead to dimerization with endogenous rfp, thereby interfering with normal unknown function of rfp.

In the TRP-1/ret transgenic mouse lines, tumors and proliferative activity were observed in the RPE, but proliferation started more often in the anterior part (Figs. 3, 5*b*). This might be explained by increased expression levels, earlier onset of expression or absence of an overlaying functional neuroretina. At birth, melanization in the RPE is mainly terminated and, hence, expression of TRP-1 (and tyrosinase and TRP-2) does cease thereafter. Therefore, the expression of the transgene might not be sufficient to cause tumor formation at all sites of the RPE. In transgenic mice carrying the *RET/PTC1* transgene, controlled by the 5′ region of the *H4* gene, only a limited number of tumor types (derived from mammary or sebaceous glands) was observed, even though higher expression levels were detected in other tissues (Portella *et al.,* 1996). Here, the authors suggested that RET/PTC1 was able to transform only certain cell types, in particular glandular structures. In neuroblastoma cells, activated RET can suppress proliferation and results in acquisition of a differentiated phenotype (D'Alessio *et al.,* 1995). Hence, reduction or absence of tumor formation in certain parts of the RPE would indicate that these areas are less susceptible to the activation of ret and only react by forming a stratified epithelial layer. In the tumors (Fig. 5), certain RPE-derived cells show production of pigment while neighboring RPE is unpigmented due to prevention of normal differentiation. However, some cells in the tumor may have ceased expression of the transgene, stopped proliferation and begun to reestablish characteristics of RPE cells, such as pigment production, similar to reports on physical injuries of the RPE (for review see Zhao *et al.,* 1997).

The TRP-1 promoter was effective in targeting genes specifically to the RPE of the developing eye. In TRP-1/DT-A transgenic mice, ablation of the RPE resulted in disorganization of the neuroretina. Retinal layers were maintained in places adjacent to pigmented areas of the RPE (Raymond and Jackson, 1995). In TRP-1/Tag (SV40 T antigen) transgenic mice, tumors developed from the RPE without affecting early development of this layer (Penna *et al.,* 1998). In contrast with the tumors observed in the TRP-1/ret transgenic mice, eye tumors in the TRP-1/Tag transgenic mice were malignant and invaded along the optic nerve and into the brain. Moreover, TRP-1/Tag RPE tumor cells metastasized to inguinal lymph nodes and spleen. It is known that expression of SV40 T antigen can cause alterations that are irreversible and lead to maintenance of the transformed state. Thus, it is conceivable that ret expression in the RPE is not sufficient or not sufficiently high to induce similar changes. This might explain why the tumors stay benign and do not affect survival of transgenic mice.

In summary, our results show that TRP-1/ret transgenic expression prevents normal differentiation of the RPE and induces proliferation and tumor formation of the RPE. These changes in the RPE lead to microphthalmia and severely affect eye development. This is important and demonstrates that a normally developed RPE is absolutely required for eye development and eye structure. In addition, these mice might be useful models for defects of human eye development, *i.e.,* blindness, eye tumor formation and microphthalmia.

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